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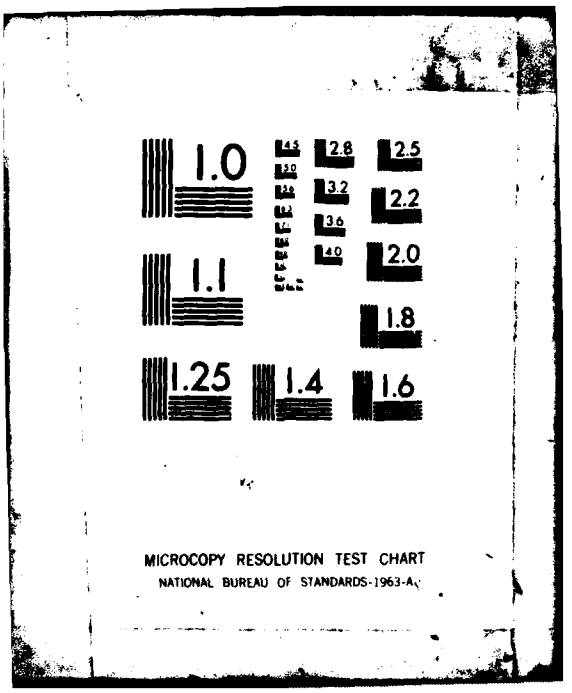
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STUDIES ON THE MECHANISM OF ACTION OF THE IN VITRO PGB_x EFFECT

IV. THE EFFECT OF ORDER OF ADDITION OF PGB_x TO ASSAY SYSTEM

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The mechanism of action of PGB_x in RLM oxidative phosphorylation was studied by varying the sequence of addition of PGB_x. When PGB_x was added to RLM pre-exposed to hypotonic media at 27° no restoration of phosphorylation was observed. When RLM were exposed to hypotonic conditions for 30 seconds before the addition of PGB_x and subsequently exposed to hypotonic conditions, the resulting phosphorylation was 60% of that observed in the normal system. From these results it is concluded that PGB_x functions in the in vitro PGB_x RLM assay system as a "protective" agent rather than a "restorative" agent.

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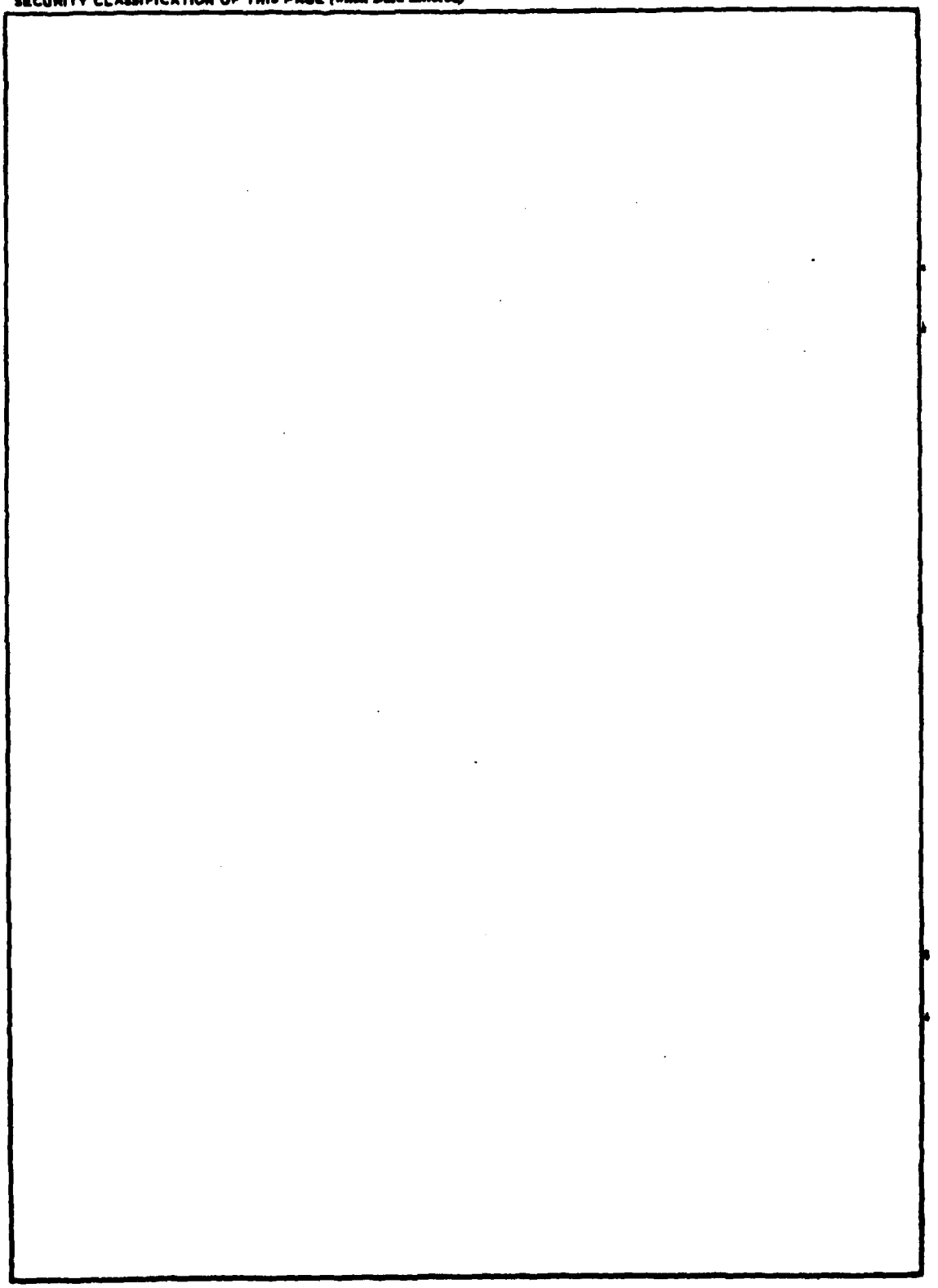

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INTRODUCTION

The possible use of PGB_X (1, 2, 3) as a therapeutic agent in the treatment of human ischemic diseases was suggested by the favorable results from only a few animal experiments (4, 5, 6, 7, 8, 9, 10). Obviously before any human trials may be undertaken much more animal testing must be completed, and in addition, certain basic information concerning the mechanism of action of this drug must be known. At this stage of our knowledge of PGB_X biochemistry, the possibility of successful elucidation of the *in vivo* mechanism of action appears remote. However, since the elucidation of the *in vitro* mechanism of PGB_X might reveal an insight as to the *in vivo* mechanism, studies have been underway in this laboratory to define the *in vitro* PGB_X effect.

Recently we reported that PGB_X ineracts with BSA (11) to form a complex that was inactive in the *in vitro* PGB_X assay system (2, 3). Some of the experimental evidence published in that report was based on altering the sequence of the addition of PGB_X and BSA in the assay system so that the PGB_X and BSA could react before the addition of RLM. The results of these experiments then suggested the importance of the sequence of addition of the reactants of the test system, and that by studying this in detail some information concerning the mechanism of the *in vitro* PGB_X effect might be realized.

EXPERIMENTAL

Materials and Methods: PGB_X Type II was synthesized and assayed for the *in vitro* PGB_X effect as reported by Polis *et al* (2, 3). RLM was isolated and stored at 0^o as described previously (2, 3). In this study the RLM were aged three days before use. All analytical methods used in this study were described previously (2, 3).

Note: Abbreviations used in this report are: RLM, rat liver mitochondria; BSA, bovine serum albumin; Pi, inorganic phosphate

Results: The effect of varying the order of addition of PGB_X to the in vitro PGB_X assay system was studied by measuring the changes in phosphorylating ability of RLM under various experimental conditions. Table I lists the composition and experimental conditions for the in vitro PGB_X assay system (2). In the normal PGB_X assay system, RLM were added to the hypotonic medium (Mixture A, Table I) containing the requisite amount of PGB_X, and the mixture then incubated for the required time period. Phosphate acceptor (Mixture B, Table I) was then added and the mixture again incubated for 20 minutes. At the end of this time period the solution was deproteinized and the Pi esterfied was measured. The extent of phosphorylation by this system was compared to that resulting from the following changes in experimental protocol: (a) RLM were incubated in Mixture A in the absence of PGB_X for the required time period; at the end of this incubation period PGB_X was added followed by the addition of Mixture B; the assay was then continued as above. (b) RLM was added to Mixture A and incubated for 30 seconds; PGB_X was then added and the hypotonic incubation continued for the required time period; at the end of the incubation period Mixture B was added and the assay continued as above. Figure I shows the results of these tests. The curves shown are described in the legend of the figure. The normal assay curve of PGB_X concentration shows the usual biphasic response with the maximum phosphorylation in the range of 2-10 µg/ml of reaction. When RLM were incubated in Mixture A in the absence of PGB_X, the subsequent addition of PGB_X did not restore the phosphorylation ability of the degraded RLM. When PGB_X was added to the RLM incubated in Mixture A for 30 seconds, a biphasic phosphorylation curve was observed, however the maximum phosphorylation ability was only about 2/3 of the normal assay and in addition was shifted so that 10-20 µg PGB_X/ml reaction were required.

DISCUSSION

In earlier reports on the physiological mechanism of PGB_X action in both in vitro and in vivo systems it was suggested that PGB_X functioned to reactivate damaged mitochondria (1, 2, 3, 8, 12) and thus reverse the pathological effects of ischemia. This interpretation appears contrary to the results reported in this study with isolated RLM subjected to hypotonic degradation. The results reported here show that RLM exposed to hypotonic media at 27° show a reduced capacity for oxidative phosphorylation. However when RLM are exposed to hypotonic media at 27° containing the required amount of PGB_X, they maintain their capacity for oxidative phosphorylation. This phenomenon is the in vitro PGB_X effect on RLM. In contrast when RLM are exposed to hypotonic media at 27°, the subsequent addition of PGB_X has no effect on the recovery of phosphorylation activity of these RLM.

From the above results it may be concluded that PGB_X functions in the in vitro assay system as a "protective" agent for RLM capacity for phosphorylation, rather than a "restorative" agent that reverses the degradative effects of exposure of RLM to hypotonic conditions.

TABLE I

The Composition of the Medium for the Demonstration of the PGB_x Effect on Mitochondrial Oxidative Phosphorylation

Order of Addition	Mitochondrial Degrading Medium	Reaction Mixture
Water	1.55 ml	1.55 ml
Phosphate Buffer pH 7.35	4.98 mM	4.55 mM
α-Ketoglutarate pH 7.35	14.93 mM	13.64 mM
$MgSO_4$	4.98 mM	4.55 mM
Aged Mitochondria	1.99 mg/ml	1.82 mg/ml
Sucrose*	5.97 mM	5.45 mM
EDTA*	0.010 mM	0.009 mM
AMP	-----	2.27 mM
ADP	-----	2.27 mM
KCl	-----	45.45 mM
Bovine Serum Albumin	-----	0.68 mg/ml

Total Volume: 2.20 ml
Temperature: 28°

Degradation Time: 5-20 minutes
Reaction Time: 20 minutes

*Added with mitochondria

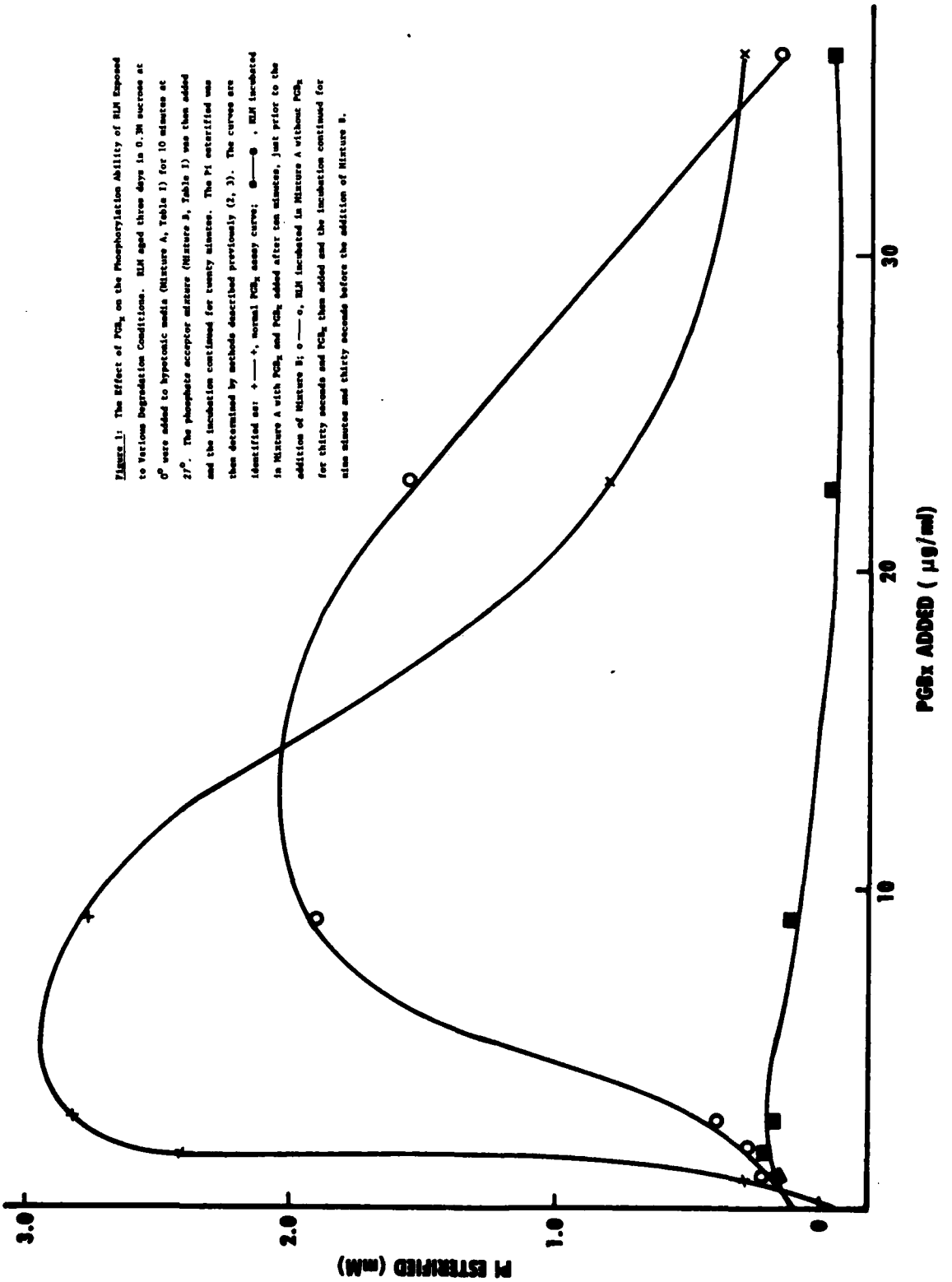

Figure 1: The Effect of PCB_x on the Phosphorylation Ability of RLM Exposed to Various Degradation Conditions. RLM aged three days in 0.3M sucrose at 0° were added to hypotonic media (Mixture A, Table I) for 10 minutes at 27°. The phosphate acceptor mixture (Mixture B, Table I) was then added and the incubation continued for twenty minutes. The PI esterified was then determined by methods described previously (2, 3). The curves are identified as: +——+, normal PCB_x assay curve; ●——●, RLM incubated in Mixture A with PCB_x and PCB_x added after ten minutes, just prior to the addition of Mixture B; o——o, RLM incubated in Mixture A without PCB_x for thirty seconds and PCB_x then added and the incubation continued for nine minutes and thirty seconds before the addition of Mixture B.

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